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Evidence for the binding of two substrate water molecules in the S_2 state of photosystem II.

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Introduction

Several unanswered questions remain regarding the mechanism of water oxidation by photosystem II (PSII). Among these are how and at what point in the reaction sequence do the two substrate-water molecules bind to the catalytic site and how does the O-O bond form. One experimental approach to address these questions is to measure the oxygen isotope exchange between the substrate water bound at the catalytic site and the solvent water using mass spectrometric techniques. The procedure involves the rapid transfer of PSII-containing samples from solvent water of known oxygen isotope composition (eg. ^{16}O-enriched water) into solvent water of another oxygen isotope composition (eg. ^{18}O-enriched water). The rate of isotope incorporation into the photogenerated O_2 is then determined as a function of the exchange time and is related to the substrate binding affinity.

By using a 'closed' sample chamber system, we have been able to measure isotope exchange times in the order of 10 ms, which is a 5000-fold improvement over earlier measurements. Thus, we are able to measure fairly rapid oxygen isotope exchange reactions. In the mass spectrometric measurement of O_2 produced by PSII, the signal at *m/e* = 34 monitors the mixed labelled $^{16}O^{18}O$ product. Since two water molecules react to form molecular O_2, the measurement at *m/e* = 34 will be sensitive to the independent exchange at the two substrate sites. Indeed, when the rate of ^{18}O incorporation into molecular O_2 from $H_2{}^{18}O$ injected into the S_3 state of spinach thylakoids was measured, strong biphasic kinetics were obtained (Hillier *et al*., 1998). In contrast, when the signal at *m/e* = 36 for the doubly labelled $^{18}O^{18}O$ product was measured, only a single kinetic phase was observed. The rate constant for the *m/e* = 36 data was virtually identical to the rate constant determined for the slow kinetic phase in the *m/e* = 34 data (Hillier *et al*., 1998). These results clearly indicated that the substrate water binds to separate sites within the water oxidizing complex in the S_3 state and that the overall incorporation of ^{18}O into molecular O_2 is limited by the substrate water undergoing the slowest isotope exchange process.

When the ^{18}O exchange was measured for the S_0, S_1, and S_2 states, strong biphasic kinetics in the *m/e* = 34 data were also observed (Hillier & Wydrzynski, 2000). These results indicated that the substrate water is bound to separate sites throughout the S-state cycle and that the O-O bond forms only during the S_3 to [S_4] to S_0 transition. However, the fast phase kinetics in the S_0, S_1, and S_2 states could not be resolved by the experimental set-up used, and left open the possibility that the second substrate water molecule may only bind to the catalytic site after the formation of the S_3 state.

Recently, it has been suggested that the fast phase exchange kinetics in the S_3 state reflects substrate entry into the water oxidizing complex, in order to accommodate the idea that a Ca ion acts as substrate-water binding site (Vrettos *et al*., 2001). By introducing an accessibility barrier, the fast 1-2 ns exchange times typical of Ca-OH_2 complexes (Hillier & Wydrzynski, 2001) could then be reconciled with the much slower fast phase kinetics. In this connection it is commonly viewed that the three extrinsic proteins of 17, 23, and 33 kDa apparent molecular mass constitutes an accessibility barrier between the catalytic site and the solvent water. In our study on the influence of PSII peripheral polypeptides on the oxygen isotope exchange reactions in the S_3 state (Hillier *et al*., 2001), we identified a set of conditions in which the fast phase kinetics in the S_2 state could be resolved. The present communication reports this finding.

Materials and Methods

PSII-enriched membrane fragments were prepared from fresh spinach thylakoids using standard procedures (Berthold *et al*., 1981) and resuspended in a buffer medium consisting of 30 mM MES (pH 6.3), 400 mM sucrose, 15 mM NaCl, and 5 mM $MgCl_2$. The extrinsic 17 and 23 kDa proteins were depleted by incubating PSII–enriched membranes in 1 M NaCl at 1 mg Chl mL^{-1} for two separate 30 min treatments in the dark on ice. The samples were centrifuged between each treatment and then finally washed twice in the standard buffer medium.

^{18}O isotope incorporation into the O_2 produced by the PSII samples was determined at *m/e* = 34 with an in-line mass spectrometer (VG MM6, Winsford, UK). An enclosed, stirred chamber was connected to the mass spectrometer via a dry ice/ethanol ice bath and injection of $H_2{}^{18}O$ was achieved using a solenoid-activated Hamilton CR-200 syringe. Samples were illuminated with saturating light flashes provided by a xenon flash lamp (EG&G FX193, 4 µF @ 1 kV capacitor) coupled to a fiber optic situated directly in front of the sample chamber window. The flash and injection protocols for the S_3 and S_2 states are described earlier (Hillier & Wydrzynski, 2000).

Results and Discussion

Table 1 reports the steady-state rates of O_2 evolution for the variously treated samples of PSII-enriched membrane fragments. Upon the removal of the extrinsic 17 and 23 kDa proteins by treatment with 1 M NaCl, the activity drops to 23% of the control, as expected. Incubation of the polypeptide-depleted sample in 15 mM $CaCl_2$ for 1-2 h restores the activity to 90% of the control, whereas incubation in 15 mM $MgCl_2$ has no effect on the residual activity. The addition of either 15 mM $CaCl_2$ or $MgCl_2$ to the control samples has little influence on the activities.

Table 1 O_2 evolution activities and ^{18}O exchange rates for the variously treated PSII samples.

Sample[a,b]	Activity[d]	Percent Control	S_3 State		S_2 State	
			$^{34}k_1$ (s^{-1})	$^{34}k_2$ (s^{-1})	$^{34}k_1$ (s^{-1})	$^{34}k_2$ (s^{-1})
Control	862 ± 63	100%	2.5 ± 0.6	30.1 ± 1.6	1.5 ± 0.3	>175
+ $CaCl_2$	834 ± 19	97%	n.d[e]	n.d.	n.d.	n.d.
+ $MgCl_2$	875 ± 37	101%	n.d.	n.d.	n.d.	n.d.
Salt-Washed[c]	200 ± 6	23%	2.4 ± 0.6	20.3 ± 3.5	2.9 ± 0.9	117 ± 7.9
+ $CaCl_2$	775 ± 22	90%	2.0 ± 0.7	17.9 ± 3.9	1.8 ± 0.4	90.3 ± 5.2
+ $MgCl_2$	208 ± 21	24%	2.3 ± 0.7	26.9 ± 5.7	1.6 ± 0.3	74.6 ± 9.3

[a] Triton X-100 prepared PSII-enriched membrane fragments, see text
[b] $CaCl_2$ and $MgCl_2$ added to a final concentration of 15 mM and incubated for 1-2 h
[c] Samples treated with 1 M NaCl to remove 17 and 23 kDa extrinsic proteins, see text
[d] [μmol O_2 (mg Chl)$^{-1}$ hr^{-1}]
[e] not determined

Table 1 also reports the rate constants for the slow ($^{34}k_1$) and fast ($^{34}k_2$) fast phases of exchange determined from kinetic plots of the normalized O_2 yield measured at *m/e* =34 as a function of isotope exchange time in the S_3 and S_2 states. Analysis of the data is described in Hillier & Wydrzynski (2000). As previously reported (Hillier *et al*., 2001), both $^{34}k_1$ and $^{34}k_2$ for the PSII-enriched membrane fragments in the S_3 state remain virtually the same as found in spinach thylakoids (data not shown), indicating that treatment with Triton X-100 does not affect the substrate exchange properties in the S_3 state. Similarly, $^{34}k_1$ in the S_2 state is also nearly the same for both the PSII-enriched membrane fragments and spinach thylakoids (and is comparable to the $^{34}k_1$ in the S_3 state), while as found earlier, the $^{34}k_2$ in the S_2 state cannot be resolved by our current experimental set-up. This last observation has been taken to indicate that the second substrate water molecule may only bind to the catalytic site after the formation of the S_3 state (Hillier & Wydrzynski, 2000).

Upon treatment of the PSII-enriched membrane fragments with 1 M NaCl, $^{34}k_1$ and $^{34}k_2$ in the S_3 state are similar to what are found in the untreated control sample (within experimental variation), except perhaps for a small slowing down in $^{34}k_2$. However, in the S_2 state, although $^{34}k_1$ turns out to be the same as the control, $^{34}k_2$ has slowed down considerably, so that it can be resolved by the current experimental set-up. Interestingly, after the addition of 15 mM $CaCl_2$ to the salt washed sample, which restores two thirds of the original O_2 evolution activity, the $^{34}k_2$ becomes if anything somewhat slower, not faster. The addition of 15 mM $MgCl_2$ to the salt-washed samples results in little effect on either the O_2 evolution activity or the exchange rates. Thus, upon removal of the 17 and 23 kDa extrinsic proteins by NaCl treatment, the fast phase exchange in the S_2 state is specifically slowed down. Clearly, this result shows that the second substrate water molecule must be bound to the catalytic site under these conditions.

The effects of Ca on the above measurements are however complex. Although we did not use specific ionophores and chelators to remove the functional Ca (Vrettos *et al*., 2001), there is clearly a significant (~65%) Ca-specific restoration of O_2 evolution activity in the NaCl

treated samples. If the simple salt washing treatment that we used leads to the loss of the functional Ca, then the exchange results indicate that Ca is unlikely to be a substrate binding site. For if it were, we would have expected the $^{34}k_2$ in the S_2 state to get faster upon Ca restoration, comparable to what is observed in the untreated control (clearly there is little effect of Ca on either the $^{34}k_1$ or the $^{34}k_2$ in the S_3 state). On the other hand, if the simple salt washing does not remove the functional Ca, then there must be another major effect of Ca on the O_2 evolution activity, outside of its role as a cofactor in the water oxidation reaction. However, this effect is not an ionic strength phenomenon, as Mg does not restore the O_2 evolution activity under these conditions. Whatever this effect is, it must be taken into consideration in the analysis of the Ca requirement for water oxidation based on steady state O_2 evolution measurements.

The present results also have another important implication for the water oxidation mechanism. After treatment with NaCl the exchange rates if anything slow down. The slowing is particularly pronounced in $^{34}k_2$ in the S_2 state. Thus, it is unlikely that the 17 and 23 kDa extrinsic proteins forms a type of accessibility barrier between the solvent water and the catalytic site, since we would expect the exchange rates to get faster upon the removal of an accessibility barrier.

The question becomes as to how do the 17 and 23 kDa extrinsic proteins and Ca interact to influence the substrate exchange reactions. The effects of polypeptide removal and Ca addition on the exchange rates are relatively small and probably do not reflect changes in the oxygen ligand structure at a metal site. Rather, these effects most likely arise from subtle outer sphere perturbations. One possibility is that the various treatments affect solvent penetration and the water structure around the catalytic site. The consequent changes in the local dielectric could then influence the exchange reactions at the substrate binding sites.

References

Berthold, D.A., Babcock, G.T, Yocum, C.F. (1981) *FEBS Lett.* **134**, 231-234.
Hillier, W., Messinger, J., Wydrzynski, T. (1998) *Biochemistry* **37**, 16908-16914.
Hillier, W., Wydrzynski, T. (2000) *Biochemistry* **39**, 4399-4405.
Hillier, W., Wydrzynski, T. (2001) *Biochim. Biophys. Acta* **1503**, 197-209.
Hillier, W., Hendry, G., Burnap, R., Wydrzynski, T. (2001) *J. Biol. Chem*., submitted
Vrettos, J.S., Stone, D.A., Brudvig, G.W. (2001) *Biochemistry* **40**, 7937-7945.